

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07K 13/00, C12N 15/00 A61K 37/02	A1	(11) International Publication Number: WO 94/10199 (43) International Publication Date: 11 May 1994 (11.05.94)
(21) International Application Number: PCT/US92/08317 (22) International Filing Date: 30 October 1992 (30.10.92) (71) Applicant: MCGILL UNIVERSITY [CA/CA]; Office of Technology Transfer, 3550 University Street, Montreal, Quebec H3A 2A7 (CA). (71)(72) Applicant and Inventor: MURGITA, Robert, A. [US/CA]; 17 Parkside Place, Montreal, Quebec H3H 1A7 (CA). (74) Agent: CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE). Published <i>With international search report.</i>

(54) Title: EXPRESSION AND PURIFICATION OF CLONED HUMAN ALPHA-FETOPROTEIN**(57) Abstract**

In general, the invention features a method for producing human alpha-fetoprotein in prokaryotic cells. The method includes: providing a transformed prokaryotic cell including a recombinant DNA molecule encoding human alpha-fetoprotein operably linked to sequence elements capable of directing expression of alpha-fetoprotein, and permitting the transformed cell to express human alpha-fetoprotein.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TC	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

- 1 -

**EXPRESSION AND PURIFICATION OF CLONED
HUMAN ALPHA-FETOPROTEIN**

Background of the Invention

5 The field of the invention is expression and purification of cloned human alpha-fetoprotein.

 Alpha-fetoprotein (AFP) is a serum protein normally found at significant levels only in fetal blood. In adult blood increased alpha-fetoprotein levels are
10 associated with liver regeneration and certain carcinomas.

 The specific function of alpha-fetoprotein is not known. Suggested roles for the protein include: fetal albumin; protection from maternal immune attack; and
15 protection from maternal estrogen.

 Morinaga et al. (*Proc. Natl. Acad. Sci. USA* 80:4604, 1983) report the cloning of human AFP.

 Innis et al. (*Arch. Biochem. Biophys.* 195:128, 1979) report the cloning of an approximately 950 base-
20 pair fragment of human AFP into *E. coli* plasmid pBR322.

 Nishi et al. (*J. Biochem.* 104:968, 1988) report the expression of rat AFP in *E. Coli* and *Saccharomyces cerevisiae*. Nishi et al. also report that, in an estradiol-binding assay, yeast-produced rat rAFP is as
25 active as authentic AFP, while bacterial-produced rat rAFP is essentially inactive. Further, when characterized by radioimmunoassay or an Ouchterlony double immunodiffusion assay, yeast-produced rat rAFP bears a closer resemblance to authentic rat AFP than does bacterial-produced rat
30 rAFP. Nishi et al. state that:

 "In the Ouchterlony double immunodiffusion test, authentic and yeast rAFP formed a completely fused precipitin line with antibody to rat AFP while *E. coli* rAFP
35 showed a reaction of partial identity in a similar test It is likely that the functionally active yeast rAFP in this

- 2 -

study had the correct pairs of disulfide bridges. On the other hand the *E. coli* rAFP probably failed to form them".

Yamamoto et al. (*Life Sciences* 46:1679, 1990)

5 report the expression of human AFP in yeast and report that the rAFP so produced was "indistinguishable immunologically from authentic AFP."

Giuliani et al. (*Protein Engineering* 2:605, 1989)

10 report the expression of a portion of human AFP (amino acids 38 to 119) in *E. coli*.

Japanese Patent Application 88158596 reports a method for preparing recombinant human domain I AFP in *E. coli*.

Summary of the Invention

15 In general, the invention features a method for producing human alpha-fetoprotein in a prokaryotic cell. The method includes providing a transformed prokaryotic cell that includes a recombinant DNA molecule encoding human alpha-fetoprotein operably linked to an expression
20 control element capable of directing expression of human alpha-fetoprotein, and permitting the transformed cell to express human alpha-fetoprotein. In a preferred embodiment, the prokaryotic cell is *E. coli*. In more preferred embodiments, the expression control element
25 includes an *E. coli* Trp promoter and the expression control element includes an *E. coli* Tac promoter.

In a related aspect, the invention features substantially pure human alpha-fetoprotein produced by providing a transformed prokaryotic cell that includes a
30 recombinant DNA molecule encoding human alpha-fetoprotein operably linked to an expression control element capable of directing expression of human alpha-fetoprotein, and permitting the transformed cell to express human alpha-fetoprotein.

- 3 -

In a related aspect, the invention features a therapeutic composition comprising substantially pure alpha-fetoprotein produced as described above.

By "human alpha-fetoprotein" is meant a polypeptide having substantially the same amino acid sequence as the protein encoded by the human alpha-fetoprotein gene. Moringa et al. (*Proc. Natl. Acad. Sci. USA* 80:4604, 1983) reports the sequence of cDNA complementary to human alpha-fetoprotein.

By "expression control element" is meant a nucleotide sequence which includes recognition sequences for factors that control expression of a protein coding sequence to which it is operably linked. Accordingly, an expression control element generally includes sequences for controlling both transcription and translation, for example, promoters, ribosome binding sites, repressor binding sites, and activator binding sites.

By "substantially the same amino acid sequence" is meant a polypeptide that exhibits at least 80% homology with naturally occurring amino acid sequence of human alpha-fetoprotein, typically at least about 85% homology with the natural human alpha-fetoprotein sequence, more typically at least about 90% homology, usually at least about 95% homology, and more usually at least about 97% homology with the natural human alpha-fetoprotein sequence. The length of comparison sequences will generally be at least 16 amino acids, usually at least 20 amino acids, more usually at least 25 amino acids, typically at least 30 amino acids, and preferably more than 35 amino acids.

Homology, for polypeptides, is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Protein analysis

- 4 -

software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

As used herein, the term "substantially pure" describes a protein or polypeptide which has been separated from components which naturally accompany it. Typically, a protein of interest is substantially pure when at least 60% to 75% of the total protein in a sample is the protein of interest. Minor variants or chemical modifications typically share the same polypeptide sequence. A substantially pure protein will typically comprise over about 85 to 90% of the protein in sample, more usually will comprise at least about 95%, and preferably will be over about 99% pure. Normally, purity is measured on a chromatography column, polyacrylamide gel, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from the native contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Thus the term can be used to describe polypeptides and nucleic acids derived from eukaryotic organisms which have been synthesized in *E. coli* and other prokaryotes.

The present invention provides for substantially pure human alpha-fetoprotein. Various methods for the isolation of human AFP from biological material may be devised, based in part upon the structural and functional

- 5 -

properties of human alpha-fetoprotein. Alternatively, anti-AFP antibodies may be immobilized on a solid substrate to generate a highly specific affinity column for purification of human AFP.

5 Besides substantially full-length polypeptides, the present invention provides for biologically active recombinant fragments of alpha-fetoprotein. For example, fragments active in ligand binding or immunosuppression.

 The natural or synthetic DNA fragments coding for
10 human alpha-fetoprotein or a desired fragment thereof will be incorporated into DNA constructs capable of introduction to and expression in cell culture. DNA constructs prepared for introduction into such hosts will typically include an origin of replication which can be
15 utilized by the host cell, a DNA fragment encoding the desired portion of human alpha-fetoprotein, transcription and translational initiation regulatory sequences operably linked to the alpha-fetoprotein encoding segment, and transcriptional and translational
20 termination regulatory sequences operably linked to the alpha-fetoprotein encoding segment. The transcriptional regulatory sequences will typically include a heterologous promoter which is recognized by the host. The selection of an appropriate promoter will depend upon
25 the host, but promoters such as the trp, tac and phage promoters, tRNA promoters and glycolytic enzyme promoters may be used under appropriate circumstances (Sambrook et al. eds., *Molecular Cloning: Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY 1989). In
30 some instances it may be desirable to include appropriately positioned recognition sequences for factors capable of regulating transcription in the host cell (e.g., the lac repressor of *E. coli*). Commercially available expression vectors, which include the
35 replication system and transcriptional and translational

- 6 -

regulatory sequences together with convenient sites for the insertion of a DNA fragment encoding the gene to be expressed may be used.

5 The various promoters, transcriptional, and translational described above are generally referred to as an "expression control element".

It is also possible to integrate a DNA fragment encoding all or part of human AFP into the host cell's chromosome.

10 The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host (Sambrook et al., supra). The term "transformed cell" is meant to also include the progeny
15 of a transformed cell.

Prokaryotic hosts useful for high level expression of recombinant proteins include: various strains of *E. coli*, *Bacillus subtilis*, and *Pseudomonas*.

20 The method of the invention provides a means by which to generate large quantities of human alpha-fetoprotein having biological activity. AFP produced according to the method of the invention has biological activity despite the fact that it is not modified in the same fashion as naturally occurring human AFP.

25 Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

Construction of a cDNA Library

30 A cDNA library was constructed with size-fractionated cDNA (0.5-3 kb) prepared from poly(A)⁺ RNA isolated from liver cells (~3 grams wet weight) of a 4.5 months old human abortus. (Alternatively, a fetal cDNA library may be obtained from Clontech Laboratories, Inc.,

- 7 -

Palo Alto, CA.) Total RNA was prepared by the guanidium thiocyanate method (Chirgwin et al., *Biochemistry* 18:5294, 1979), and mRNA was selected by oligo(dT)-cellulose chromatography (Collaborative Research, Bedford, MA) (*Current Protocols in Molecular Biology*, Ausubel et al., eds., Wiley Interscience, New York, 1989). cDNA was synthesized using the Librarian II cDNA synthesis kit (Invitrogen, San Diego, CA) and fractionated on a 1% agarose gel. Fragments of 0.5 to 3 kb were extracted and ligated to vector pTZ18-RB (Invitrogen), and used to transform competent *E. coli* DH1 α F' (Invitrogen). Colony lifts were performed with Colony/Plaque Screen filters (DuPont, Wilmington, DE), and the transferred bacterial colonies were lysed and denatured by incubation in a solution of 0.5M NaOH, 1.5M NaCl for 10 min. The filters were washed for 5 min in 1.5M NaCl, 0.50M Tris-HCl (pH 7.6), and air dried. Filters were then washed 5 times in chloroform, soaked in 0.3M NaCl to remove cellular debris, and then air dried. The DNA was fixed to the nitrocellulose by baking at 80° under vacuum for 2 hrs. The baked filters were prehybridized for 3 hr at 37°C in 6X SSC (1X SSC = 150mM NaCl, 15mM sodium citrate [pH 7.0]), 1X Denhardt's solution (0.2 g/l polyvinylpyrrolidone, 0.2 g/l BSA, 0.2 g/l Ficoll 400), 0.05% sodium pyrophosphate, 0.5 % SDS, and 100 μ g/ml *E. coli* DNA. Hybridization was performed for 18-24 hr at 37°C in the same solution without SDS, containing 1-2 x 10⁶ cpm/ml of two oligonucleotides ³²P-labelled by 5'-end phosphorylation (*Current Protocols in Molecular Biology*, *supra*). The sequence of the oligonucleotides used for probing the library: 5'-TGTCTGCAGGATGGGGAAAAA-3' (SEQ ID NO: 1) and 5'-CATGAAATGACTCCAGTA-3' (SEQ ID NO: 2), correspond to positions 772 to 792 and positions 1405 to 1422 of the human AFP coding sequence respectively. Filters were

- 8 -

washed twice for 30 min at 37°C with 6X SSC, 0.05% sodium pyrophosphate and once for 30 min at 48°C in the same solution. Dried filters were exposed to Kodak XAR films in the presence of Du Pont Cronex Lightning Plus intensifier screens for 24-48 hr to identify positive clones. Positive clones were isolated, amplified, and subjected to Southern blot analysis (*Current Protocols in Molecular Biology, supra*). Briefly, purified DNA was hydrolyzed with the appropriate restriction enzymes, and the resulting fragments were resolved on a 1% agarose gel. The DNA was then transferred to a nitrocellulose membrane. Hybridization conditions were as described above except that a third ³²P-labelled oligonucleotide (5'-CATAGAAATGAATATGGA-3' (SEQ ID NO: 3), representing positions 7 to 24 of the human AFP coding region) was used in addition to the other two probes described above. Five positive clones were identified among the 3,000 colonies screened. One clone, pLHuAFP, was used in the construction described below.

- 9 -

Construction of Full Length Human AFP cDNA

A construct containing a translation initiation codon followed by the human AFP coding sequence and a translation termination codon was created using the following five DNA fragments.

Fragment 1: Two unphosphorylated oligonucleotides were annealed to form a double-stranded DNA molecule consisting of a 5'-end cohesive EcoRI recognition site, followed by an ATG initiation codon and the first 60 bp of the human AFP cDNA up to and including the PstI restriction site located at position 60 in the coding sequence (In this scheme, nucleotide 1 is the first nucleotide of the first codon (Thr) in the mature protein and corresponds to nucleotide 102 of Morinaga et al., supra). This fragment was ligated to pUC119 (pUC19 with the intergenic region of M13 from HgiA I at 5465 to AhaII at 5941 inserted at the Nde I site of pUC19) linearized with Eco RI and Pst I. The resulting DNA was amplified in *E. coli* NM522 (Pharmacia, Piscataway, NJ). The EcoRI-PstI insert was recovered by enzymatic digestion of the recombinant plasmid followed by electrophoretic separation on a 5% polyacrylamide gel and isolation from the gel.

Fragment 2: A 97 bp human AFP cDNA fragment (positions 57 to 153) was obtained by digesting pLHuAFP with PstI and NsiI and gel purifying as described above. This clone contains the entire coding region of human AFP as well as 5' and 3' untranslated sequences.

Fragment 3: A 224 bp human AFP cDNA fragment (positions 150 to 373) was obtained by digesting pLHuAFP with NsiI and AlwNI and purifying as described above.

Fragment 4: A 1322 bp human AFP cDNA fragment (positions 371 to 1692) was obtained by digesting pLHuAFP with AlwNI and StyI and purifying as described above.

- 10 -

Fragment 5: Two unphosphorylated oligonucleotides were annealed to form a 86 bp double-stranded DNA contains the human AFP sequence from position 1693 in the StyI site to the TAA termination codon that ends the AFP coding region at position 1773, followed by a cohesive BamHI site. This synthetic DNA was used without any further manipulations.

pBlueScript (StrataGene, La Jolla, CA) was completely hydrolyzed with EcoRI and BamHI, and added to ligation mixture containing the five purified fragments described above. A control ligation contained only the linearized pBluescript. Portions of these two ligation mixtures were used to transform competent *E. coli* DH5 α (GIBCO/BRL, Grand Island, NY). Recombinant plasmids were isolated from several transformants and screened by extensive restriction enzyme analysis and DNA sequencing. One recombinant plasmid was selected and termed pHuAFP. It was used for subsequent insertion of the human AFP gene into several expression vectors. pHuAFP includes a unique EcoRI-BamHI fragment that contains the complete coding sequence for human AFP in addition to an ATG start codon at the 5'-end and a TAA stop codon at the 3'-end.

AFP Expression Vectors

Successful high-level synthesis of human AFP in *E. coli* was achieved in three different expression systems. The TRP system gave direct expression. The RX1 system yielded a fusion protein containing 20 amino acids encoded by *trpE* and vector sequences. The MAL system expressed AFP fused to the *malE* gene product, a 42 kd maltose-binding protein.

TRP Expression System: The 1186 bp EcoRI-BamHI AFP encoding fragment of pHuAFP was cloned into the expression vector pTrp4 (Olsen et al., *J. Biotechnol.* 9:179, 1989) downstream of the *trp* promoter and a modified ribosome-binding site.

- 11 -

Briefly, pHuAFP was digested with EcoRI and BamHI, and the ends were filled using Klenow polymerase. The 1186 bp AFP fragment was then gel purified. pTRp4 was ClaI digested, the ends were filled using Klenow
5 polymerase, and the linearized vector was gel purified. The 1186 bp AFP fragment and pTrp4 backbone were ligated and used to transform competent *E. coli* of the following strains: DH5 α , BL21 (F.W. Studier, Brookhaven National Laboratory, Upton, NY), SG927 (American Type Culture
10 Collection, Rockville, MD: Acc. No. 39627), SG928 (ATCC Acc. No. 39628), and SG935 (ATCC Acc. No. 39623).

RX1 Expression System: Human AFP cDNA was cloned into the expression vector pRX1 (Rimm et al., Gene 75:323, 1989) adjacent to the *trp* promoter and in the
15 translation frame of TrpE. The human AFP cDNA was excised from pHuAFP by digestion with EcoRI and BamHI and cloned into suitably treated pRX1 (BioRad Laboratories, Hercules, CA). The *E. coli* strains described above and CAG456 (D.W. Cleveland, Johns Hopkins University,
20 Baltimore, MD) were then transformed with the final plasmid construction identified as pRX1/HuAFP.

MAL Expression System: AFP cDNA was inserted into the expression vector pMAL (New England Biolabs, Inc., Beverly, MA) under control of the *tac* promoter and in the
25 translation frame of MalE. Briefly, pHuAFP was hydrolysed with BamHI and the ends made blunt using Klenow polymerase. The human AFP cDNA was released from the rest of the plasmid DNA by EcoRI digestion and then gel purified. The purified fragment was ligated to
30 appropriately digested pMAL-C. A correctly oriented recombinant plasmid, designated pMAL/HuAFP, was used to transform *E. coli* DH5 α , TBI (New England Biolabs) and SG935.

- 12 -

The AFP coding region used in the construction of the three expression vectors was sequenced and found to encode full length AFP.

Expression of AFP in *E. coli*

5 Bacterial cultures were incubated at 30°C or 37°C with aeration. Overnight cultures of *E. coli* were grown in LB medium supplemented with the appropriate antibiotics as required (Tetracycline-HCl was at 50 µg/ml, and ampicillin-Na was at 100 µg/ml).

10 TRP and RX1 Expression Systems: The *trp* promoter was induced under tryptophan starvation conditions. Induction was performed in M9CA medium prepared as follows: 1 g Casamino acids (Difco Laboratories, Detroit, MI), 6 g Na₂HPO₄, 3g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl is
15 added to one liter milli-Q water (Millipore Corp., Bedford, MA), the pH adjusted to 7.4 and the solution autoclaved. The cooled medium is made 2mM MgSO₄, 0.1mM CaCl₂, and 0.2% glucose. After a 100-fold dilution of an overnight culture in M9CA supplemented with antibiotics,
20 the cells were grown at 30°C to A₅₅₀ of 0.4, harvested by centrifugation, and stored as pellets at -20°C.

 MAL Expression System: The *tac* promoter was induced with the gratuitous inducer IPTG. Overnight cultures were diluted 100-fold in LB medium supplemented
25 with antibiotics, and the cells grown at 37°C to A₅₅₀ of 0.4. IPTG was then added to a final concentration of 0.3mM, and the bacteria incubated an additional 2 hr. The cells were then harvested by centrifugation and stored as pellets at -20°C.

- 13 -

Detection of AFP Expressed in E. Coli

Analytical studies were performed to determine the expression and behavior of recombinant AFP. Cell pellets were either suspended in SDS-lysis solution (0.16M Tris-HCl [pH 6.8], 4% w/v SDS, 0.2M DTT, 20% glycerol, 0.02% bromophenol blue), boiled for 5 min, and used for analysis by SDS-PAGE or suspended in a lysis buffer consisting of 10 mM Na₂HPO₄, 30mM NaCl, 0.25% Tween 20, 10mM EDTA, 10mM EGTA and incubated with 1 mg/ml lysozyme at 4°C for 30 min prior to sonication in pulse mode for 3 x 1 min at 50% power (Sonics and Materials, Danbury, CT: model VC300 sonifier). The lysate was centrifuged at 10,000g for 20 min, and the supernatant containing soluble protein was decanted in a separate test tube and frozen at -20°C until used. The pellet containing insoluble protein was resuspended in SDS-lysis buffer, boiled for 5 min and kept at -20°C until used. Total protein released in SDS-lysis buffer, as well as soluble and pellet fractions were analyzed by SDS-PAGE and immunological detection following western blot transfer. In these studies coomassie blue stained gels were routinely scanned with a video densitometer (BioRad, model 620). This allowed a qualitative assessment of the amount of recombinant AFP produced as a percentage of total cellular protein.

Purification of AFP Expressed in the TRP System

All procedures were carried out at 4°C, unless otherwise stated. Each frozen cell pellet from a one liter culture was resuspended in 25 ml of lysis buffer A, 50mM Tris-HCl [pH 7.5], 20% sucrose, 100 µg/ml lysozyme, 10 µg/ml PMSF), and incubated for 10 min. EDTA was added to a final concentration of 35mM, and the extract allowed to stand a further 10 min. Following the addition of 25 ml of lysis buffer B (50mM Tris-HCl [pH 7.5], 25mM EDTA, 0.2% Triton X-100), the lysate was incubated an

- 14 -

additional 30 min. The cell lysate was centrifuged at 12,000g for 20 min, and the precipitate containing the recombinant AFP was washed twice with 50 ml of wash buffer (50mM Tris-HCl [pH 8.0], 10mM EDTA, 0.2% Triton X-100), followed each time by centrifugation as above. The precipitate was dissolved in 50 ml of denaturation buffer (0.1M K_2HPO_4 [pH 8.5], 6M guanidine-HCl, 0.1M 2-mercaptoethanol), sonicated, and then mixed on a Nutator (Clay Adams) for 4 hr. The solubilized extract was diluted 50-fold in 50mM Tris-HCl, 100mM NaCl, 1mM EDTA, and the recombinant AFP protein allowed to renature for 24 hr. This 50-fold dilution step is important because prior to dilution AFP appears to be microaggregated. Subsequent to dilution and reconcentration, AFP is not aggregated. The solution was concentrated 100-fold on YM10 membranes using an Amicon filtration unit, and clarified through a Millex 0.22 μ m membrane filter (Millipore). The recombinant AFP was further purified at room temperature on a Mono Q column (Pharmacia) equilibrated in 20mM Tris-HCl (pH 8.0) with bound proteins eluted using a linear gradient of 0-100% 1M NaCl, 20mM Tris-HCl (pH 8.0). Fractions were analyzed by SDS-PAGE, APAGE, and Western blotting.

Polyacrylamide Gel Electrophoresis and Western

Immunodetection Procedures

SDS-PAGE in discontinuous buffer system and alkaline-PAGE were performed according to Hames et al. (*Gel Electrophoresis of Proteins: A Practical Approach*, IRL Press, London, 1981) using the mini-Protean electrophoresis apparatus (BioRad). Immunological detection of recombinant human AFP following SDS-PAGE or APAGE was accomplished by soaking the gels in transfer buffer (12.5mM Tris-HCl, 96mM glycine, 20% methanol [pH 8.2]) for 15 min. Individual gels were then layered with an Immobilon PVDF membrane (Millipore) and sandwiched

- 15 -

between the two electrode grids of the mini-Protean transfer device (BioRad), with the gels adjacent to the cathode. The system was immersed in transfer buffer, and a 150 mA current was applied for 2 hr. Unreacted sites
5 on the Immobilon PVDF sheets were blocked in 20mM Tris-HCl (pH 7.5), 500mM NaCl, 3% gelatin for 1 hr. Rabbit anti-human AFP antiserum and goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase (BioRad) were used as the primary and the secondary antibodies,
10 respectively. The alkaline phosphatase activity was detected using 5-brome-4-chloro-3-indolyl phosphate and p-nitroblue tetrazolium (Bio-Rad).

Quantitation of AFP Expression

Recombinant human AFP was quantitated using a
15 human AFP ELISA kit (Abbott Laboratories, Chicago, IL).

AFP yield was estimated by scanning silver stained gels. When SG935 cells are transformed with the AFP encoding plasmid that employs the Trp expression system, AFP represents 2 to 5% of total cellular *E. coli* protein
20 (approximately 3-7 mg AFP per liter of culture). As described above, most AFP in the initial extract is insoluble. The above-described resolubilization procedure permits 50-60% recovery of AFP in the form of stable, semi-purified, monomeric AFP (approximate yield
25 50 mg/20 l of *E. coli*). This can be further purified to yield 25 mg of pure monomeric AFP.

N-Terminal Analysis

Automatic Edman degradations were performed using a Porton protein/peptide gas phase microsequencer with an
30 integrated customized microbore HPLC to optimize sequence. Protein sequence analysis was aided by the use of selected programs within the PC/Gene software package (Intelligenetics).

Use

- 16 -

Recombinant human AFP and fragments thereof produced by procarkotic cells can be used for diagnostic standards and for therapeutic use.

Recombinant AFP and fragments thereof can be
5 administered in an effective amount either alone or in
combination with a pharmaceutically acceptable carrier or
diluent. The polypeptides and compositions can be
administered alone or in combination with other
therapeutic agents by any convenient means, e.g.,
10 intravenously, orally, intramuscularly, or intranasally.

- 17 -

SEQUENCE LISTING**(1) GENERAL INFORMATION:**

- (i) **APPLICANT:** Murgita, Robert A.
- (ii) **TITLE OF INVENTION:** Expression and Purification
of Cloned Human Alpha-
Fetoprotein
- (iii) **NUMBER OF SEQUENCES:** 3
- (iv) **CORRESPONDENCE ADDRESS:**
- (A) **ADDRESSEE:** Fish & Richardson
(B) **STREET:** 225 Franklin Street
(C) **CITY:** Boston
(D) **STATE:** Massachusetts
(E) **COUNTRY:** U.S.A.
(F) **ZIP:** 02110-2804
- (v) **COMPUTER READABLE FORM:**
- (A) **MEDIUM TYPE:** 3.5" Diskette, 1.44 Mb
(B) **COMPUTER:** IBM PS/2 Model 50Z or 55SX
(C) **OPERATING SYSTEM:** IBM P.C. DOS (Version 3.30)
(D) **SOFTWARE:** WordPerfect (Version 5.0)
- (vi) **CURRENT APPLICATION DATA:**
- (A) **APPLICATION NUMBER:**
(B) **FILING DATE:**
(C) **CLASSIFICATION:**
- (vii) **PRIOR APPLICATION DATA:**
- (A) **APPLICATION NUMBER:** 07/767,435
(B) **FILING DATE:** 27 September 1991
- (viii) **ATTORNEY/AGENT INFORMATION:**
- (A) **NAME:** Paul T. Clark
(B) **REGISTRATION NUMBER:** 30,162
(C) **REFERENCE/DOCKET NUMBER:** 04844/002001
- (ix) **TELECOMMUNICATION INFORMATION:**
- (A) **TELEPHONE:** (617) 542-5070
(B) **TELEFAX:** (617) 542-8906
(C) **TELEX:** 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

- 18 -

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGTCTGCAGG ATGGGGAAAAA

20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CATGAAATGA CTCCAGTA

18

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CATAGAAATG AATATGGA

18

- 19 -

Claims

We claim:

1. A method for producing human alpha-fetoprotein in a prokaryotic cell, comprising
 - 5 providing a transformed prokaryotic cell comprising a recombinant DNA molecule encoding said human alpha-fetoprotein operably linked to an expression control element capable of directing expression of said human alpha-fetoprotein, and
 - 10 permitting said transformed cell to express said human alpha-fetoprotein.
2. The method of claim 1 wherein said prokaryotic cell is *E. coli*.
3. The method of claim 1 wherein said expression
 - 15 control element comprises an *E. coli* Trp promoter.
4. The method of claim 1 wherein said expression control element comprises an *E. coli* Tac promoter.
5. Substantially pure human alpha-fetoprotein produced by the method of claim 1.
- 20 6. A therapeutic composition comprising the substantially pure alpha-fetoprotein of claim 5.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08317

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 13/00; C12N 15/00; A61K 37/02

US CL :530/324,350,363,380; 435/69.1,69.6,172.1; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/324,350,363,380; 435/69.1,69.6,172.1; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Life Sciences, Volume 46, issued 1990, Ritsu Yamamoto et al., "Expression of Human Alpha-Fetoprotein in Yeast", pages 1679-1686, see entire document.	1-6
Y	Journal of Biochemistry, volume 104, issued 1988, Shinzo Nishi et al., "Expression of rat alpha-fetoprotein cDNA in <u>Escherichia coli</u> and in yeast", pages 968-972, see entire document.	1-6
Y	Proceedings of the National Academy of Science, Volume 80, issued August 1983, Tomonori Morinaga et al., "Primary structures of human alpha-fetoprotein and its mRNA", pages 4604-4608, see entire document.	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

10 August 1993

Date of mailing of the international search report

19 AUG 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

ROBERT A. WAX

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196